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Expression of *AhR*, *CYP1A1*, *GSTA1*, *c-fos* and *TGF-α* in skin lesions from dioxin-exposed humans with chloracne

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Abstract

Occupational exposure to certain polychlorinated aromatic hydrocarbons such as dioxins has been suggested to cause chloracne which is a kind of skin disease. The molecular mechanisms of dioxin-mediated chloracne have not been clarified. It is possible that dioxins contribute to the pathogenesis through activation of aryl-hydrocarbon receptor (AhR)-mediated transcription and downstream genes such as *CYP1A1*, *GSTA1* and *TGF-α*. The study on genes was through chloracne lesional skin, which has rarely been reported on previously. The expression levels of key genes, such as *AhR*, *CYP1A1*, *GSTA1*, *c-fos* and *TGF-α* in human epidermal tissue of chloracne cases and controls were detected by real-time PCR. Compared with controls, *AhR*, *CYP1A1*, *GSTA1* and *c-fos* transactivations were significantly induced in the skins of chloracne patients who had long-term exposure to dioxins and dibenzofuranes. The TGF-α mRNA content of epidermal tissue was increased, but not significantly compared with controls. The study demonstrates that constitutive activation of the AhR pathway is probably a prerequisite of chloracne pathogenesis. The changes of genes expression may disturb normal proliferation and differentiation of human epidermis cells, and then lead to chloracne.

Keywords: TCDD; Chloracne; AhR; CYP1A1; GSTA1; c-fos

1. Introduction

Dioxins are used to specify polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and dioxin-like polychlorinated biphenyls (PCBs), which can elicit a wide spectrum of toxic responses in humans including reproductive, developmental and immunologic toxicities as well as carcinogenicity (Sweeney and Mocarelli, 2000). The most biologically active isomer is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), and probably the most potent compound ever synthesized by man (Travis and Hattemer-Frey, 1991). Chloracne is regarded as a reliable indicator of heavy dioxin exposure (Neuberger et al., 1991) and is a persistent process that remains years after occupational exposure (Scerri et al., 1995). The chronicity of chloracne may relate to the fact that chloracnegens are highly lipophilic with a low rate of metabolism, leading to persistence in human adipose tissue for sustained periods. The Yusho study on chloracne cases in Japan showed whose lesions persisted longer than 30 years have a certain degree of correlation with blood dioxin level (Imamura et al., 2007). Chloracne is a well-recognized but poorly understood dermatosis. The multitude of acne-like eruption of comedones, cysts and pustules, and by squamous metaplasia of epithelial cells within the duct of the sebaceous gland are characteristic (Geusau et al., 2005; Zugerman, 1990). Although the clinical features of chloracne are clearly defined, the effective treatment for chloracne is difficult, and poor understanding of the molecular mecha-

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nism of dioxin-induced chloracne hampers a rational approach to therapy.

The pleiotropic effects caused by dioxins in humans and animals appear to be mediated by AhR (aryl hydrocarbon receptor), which was a ligand-activated transcription factor essential for inducing a battery of phases I and II xenobiotic metabolizing enzymes (XMEs), including cytochrome P450 monooxygenases (P450s) and glutathione S-transferase (GST) (Prough, 1996). P450s play an essential role in both metabolic activation and detoxification (Nebert et al., 2004). CYP1A1 is one of the most intensely studied P450s, which has proven to be a useful biomarker of exposure to dioxins in many animal species (Ramadoss and Perdew, 2005). GST are a multi-gene family of enzymes playing an important role in the detoxification of environmental toxins and products of oxidative stress, and in a few instances, activation of a wide variety of chemicals (Operana et al., 2007). GSTA1 is a member of the alpha class of GST, which is abundantly formulated in the liver and is obviously induced by dioxin through *AhR*, *TGF-α* and *c-fos* genes are probably involved in another functional TCDD-dependent pathway, which was implicated in the control of developmental processes and independent of detoxification (Choi et al., 1991; Dohr et al., 1994). In the present study, an analysis was performed to compare the expression levels of *AhR*, *CYP1A1*, *GSTA1*, *c-fos* and *TGF-α* genes in cases and controls. The goal was to clarify the effects of these genes on dioxin-induced chloracne and to provide some proof for further research.

2. Subjects and methods

2.1. Study subjects

The study protocol was approved by the Ethics Committee for Human Research, Tianjin Medical University. Informed consent was obtained from all study subjects after explanation of the nature and possible consequences of the study. Chloracne cases were obtained from employees with chloracne in a Chinese factory where pentachlorphenol (PCP) was produced from hexachlorocyclohexane (HCH) before 2002. Analysis of the production process had indicated that dioxins had come up as the inevitable impurities when trichlorobenzene was heated in the presence of oxygen, with iron as a catalyst (Cheng et al., 1993). That was the likely reason why workers in this production unit suffered from chloracne.

We recruited 12 workers (males, age 46–60 years old) with visible clinical manifestations of chloracne as the case group. We did not detect the exact PCDD and PCDF levels of the workers or control subjects. We had, however, reliable estimates of PCDD and PCDF in blood lipids of the workers (concentrations were between 1168 and 22,308 pg/g in toxicity equivalents (TEQs) in blood (Coenraads et al., 1999)). The chloracne cases of the present study were from the same workplace (with exactly the same work-circumstances) as the workers in the former study. Controls were 12 healthy age-matched males with no history of such kind of exposure or other skin disease.

2.2. Drinkers and smokers definition

In our study, drinkers were those who used alcohol (that is, wine, beer or hard liquor) on 3 or more days per week. Those who indicated that they were occasional or party drinkers were not counted as drinkers in this study. A smoker was defined as a person who had smoked as much as one cigarette a day for as long as 1 year (Doll and Hill, 1950).

2.3. Sample collection

Local anesthesia with lidocaine has been used in all subjects, according to the same procedures as used in routine clinical dermatological practice. Punch biopsies were taken in order to obtain epidermal tissue from lesional skin by Biopsy Punch (Waechtersbach, Germany) three from the neck and five from the back were obtained. We take four samples of the skin from the face by taking fusiform incision in order to suture easily and guarantee facial appearance. The controls skin was taken from the same localizations and by the same process. Every piece of epithelium was 5 mm × 2 mm in diameter × thickness. The processes above were operated by an experienced dermatology surgeon. Immediately after the collection, each skin sample was briefly washed in saline, snap-frozen in liquid nitrogen, and stored in liquid nitrogen.

2.4. RNA isolation

Total RNA was isolated from thawing the frozen samples with the TRIzol Reagent kit (TaKaRa Biotechnology, Dalian, China) according to the manufacturer's instructions. The total RNA was measured at 260 and 280 nm using an ultramicrospectrophotometer. The 260/280 nm ratios, as well as a 1% agarose-formaldehyde gel stained with ethidium bromide, were used to verify the RNA quality in each sample. The RNA concentrations were adjusted to 75 ng/μl for first-strand cDNA synthesis.

2.5. Reverse transcription

The reverse transcription was according to the Takara RT kit (TaKaRa Biotechnology, Dalian, China). Synthesis of first-strand cDNA was performed using TaqMan reverse transcription reagents immediately after the RNA had been prepared. Reverse transcription was carried out in 10× RT buffer, 25 mM magnesium chloride, 10 mM dNTP mixture, 0.4 U/μl of RNase inhibitor, 3.5 U/μl of AMV reverse transcriptase, oligo dT-adaptor primer, ≤1 μg sample RNA and RNasefree water to a final volume of 20 μl. The reaction was incubated at 42 °C for 50 min, and then the reverse transcriptase was inactivated (99 °C, 5 min). All samples were reverse transcribed in duplicate for further study by real-time polymerase chain reaction (PCR). A control without reverse transcriptase was performed in each RNA sample to confirm that no DNA contamination had occurred. *β-Actin* was used as an endogenous control.

2.6. Primer design

PCR primers for the selected genes were designed based on published sequences (GenBank, accession numbers in Table 1), and their specificity was verified with BLAST alignment search. To confirm amplification of the expected size fragment, amplification products were characterized by agarose gel electrophoresis. Identity of amplicons was further verified by the sequencing analysis (result not shown).

2.7. Real-time polymerase chain reactions

AhR, *CYP1A1*, *GSTA1*, *c-fos* and *TGF-α* cDNA were amplified by a real-time PCR detection system using SYBR® Green I as the detection dye. Amplification reactions were performed using QuantiTect SYBR Green PCR kit (catalog no. 204143, Hamburg, Germany,) with each reaction containing the same volume sample template (cDNA) production, SYBR Premix Ex TaqTM (2×), PCR upper primer (10 μM), PCR lower primer (10 μM) and dH_2O a 50 μl volume. This kit was optimal for PCR products between 100 and 600 bp. The reactions were cycled 40 times using the following parameters: 95 °C for 15 s, 53 °C (55 °C, Table 1) for 30 s and 72 °C for 30 s during which the fluorescence data were collected. At the end of the PCR, a melting curve was generated to verify the specificity of the product. A non-template control was carried out with every assay and no indication of PCR contamination was observed.

2.8. Statistical analysis

All statistical analyses were done with SPSS 11.5 with log transformation of the data. Statistical analysis of the comparison of the expression level of genes

Table 1
Sequence of primers used in real-time PCR, amplicon sizes and annealing temperatures

Targets accession no.	Sequences	Amplicon length (bp)	Annealing temperature (°C)
β-Actin, BC002409	F: 5′ CACCCCACTTCTCTCTAAGG 3′; R: 5′ AAAAAGTATTAAGGCGAAGAT 3′	126	53
AhR, NM_001621	F:5′ AACGGAGGCCAGGATAACTGTAGAG 3′; R: 5′CATCAGACTGCTGAAACCCTAGATAAT 3′	175	55
CYP1A1, M_000499	F: 5′ TAGACACTGATCTGGCTGCAG 3′; R: 5′ GGGAAGGCTCCATCAGCATC 3′	146	53
GSTA1, M_145740	F: 5′ AAGGAGAGAGCCCTGATTGATATGT 3′; R: 5′ GTCTTGTCCATGGCTCTTTAAGACT 3′	180	53
c-fos, K00650	F: 5′ GAGGGGCAAGGTGGAACAGTTATC 3′; R: 5′ GTCTGTCTCCCGCTTGGAGTGTATC 3′	137	58
TGF-α, NM_003236	F: 5′ GCCCTGGCTGTCCTTATCATCAC 3′; R: 5′ CTCTGGGCTCTTCAGACCACTGT 3′	167	59

between the cases and the controls was by Student's *t*-test, with a *P*-value of 0.05 as level of significance.

3. Results

3.1. Basic demographic characteristics of cases and controls

Cases ($n = 12$) and controls ($n = 12$) were all males, and were comparable in age (52 ± 7 vs. 52 ± 5), body mass index (BMI) (23.54 ± 2.48 vs. 24.72 ± 2.37), and smoking and drinking habits (Table 2). In our study, drinking and smoking were potential confounding factors for the relationship between dioxin existence and genes expression level. Statistical analysis have shown that the difference between the chloracne cases and controls were not statistically significant.

In addition, we presented geometric mean and range of *CYP1A1* expression levels separately for smokers and nonsmokers in the cases and the controls, respectively (Table 3). In the controls, the *CYP1A1* mRNA content of the smokers was increased without significant difference with nonsmokers. Unfortunately, almost all male workers in this factory are smokers, so we could detect only one nonsmoker among the cases. The *CYP1A1* mRNA expression difference between the cases and the controls is almost tenfolds. We could not exclude the adverse point of insufficient sample size, however, the result may illustrate that chloracne is the main effect compared to smoking during the expression alteration.

Table 2
Baseline characteristics of controls and chloracne cases

	Controls (%)	Chloracne cases (%)	*P*
Smokers			
+	9 (75.0)	11 (91.7)	0.590
−	3 (25.0)	1 (8.3)	
Drinkers			
+	10 (83.3)	8 (66.7)	0.640
−	2 (16.7)	4 (33.3)	

3.2. Transcript levels of AhR, CYP1A1, GSTA1, c-fos and TGF-α mRNA in human epidermis

The transcript levels of genes mRNA in human epidermis were evaluated by analyzing log-transformed data with a Student's *t*-test. Compared with the controls, *AhR*, *CYP1A1*, *GSTA1* and *c-fos* transactivations were induced in the skin of chloracne patients with long-term exposure to dioxins and dibenzofuranes. These gene expression amounts show significant upregulation compared with the control group ($P < 0.05$), while the *TGF-α* mRNA content of the epidermis of the cases was increased without significant difference ($P = 0.150$) (Fig. 1).

4. Discussion

Overall, we observed a notable alteration of gene expression in terms of numbers of genes altered and expression ratios. In the comparison between controls and chloracne cases, upregulation of four genes was notable in chloracne lesional skin, and was confirmed by real-time PCR.

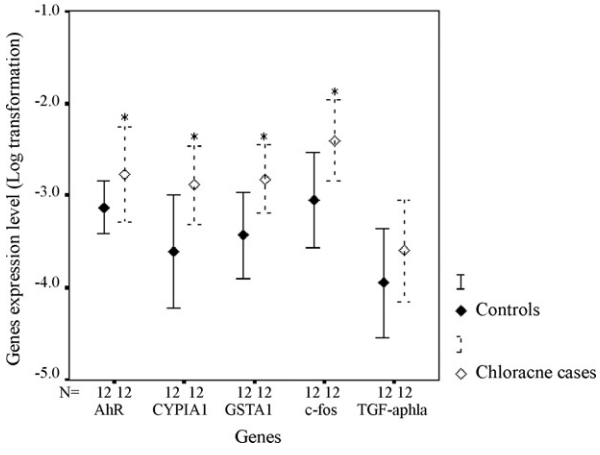

Fig. 1. Expression levels of *AhR*, *CYP1A1*, *GSTA1*, *c-fos* and *TGF-α* mRNA in chloracne cases and controls by real-time PCR. Log transformations of the data were presented as mean ± S.D. to compare the expression level of genes between the cases and the controls. Compared to the control group, significance of difference was determined at $*P < 0.05$.

Table 3
CYP1A1 expression levels for smokers and nonsmokers in controls and chloracne cases

Groups	Smoking	*n*	*CYP1A1* levels	
			Geometric mean	Range
Controls	+	9	0.000286	0.00001–0.0025
	−	3	0.000167	0.00007–0.00049
Chloracne cases	+	11	0.002266	0.0003–0.0129
	−	1	0.001230	–

AhR is considered to play not only a role in the regulation of xenobiotic metabolism, but also for development, growth, and differentiation (Monk et al., 2003). The binding of dioxins to AhR leads to receptor dimerization with aryl hydrocarbon nuclear translocator (ARNT) followed by subsequent binding of this heterodimer to the dioxin response elements (DREs) located in the promoter region of certain genes. This upregulates a battery of genes encoding for xenobiotic metabolizing enzymes (Elferink and Whitlock, 1990; Okey et al., 2005) such as cytochrome P450, glutathione S-transferase, UDP-glucuronosyl transferase, NADPH, and quinine oxidoreductase (Mimura and Fujii-Kuriyama, 2003; Panteleyev and Bickers, 2006).

The AhR/ARNT pathway plays an important role in dioxin toxicity, which has been clearly established in many target tissues such as lungs, liver and thymus. However, the role of AhR/ARNT pathway in mediating skin effects of dioxins such as chloracne is still unclear. Our study detected three key genes which were involved in the AhR/ARNT pathway: *AhR*, *CYP1A1* and *GSTA1*. The result suggests that the expression levels of *AhR*, *CYP1A1* and *GSTA1* mRNA were significantly upregulated in epidermal tissue from chloracne lesional skin.

The structural and functional characterization of AhR is critical for understanding the responses to dioxins. The high level of *AhR* expression in human epidermis and the finding that *AhR* was expressed in epidermal keratinocytes in a differentiation-associated manner (Wanner et al., 1996), supported the possible involvement of AhR in chloracne pathogenesis in humans. The essential role of many AhR/ARNT-dependent genes in the maintenance of skin homeostasis indirectly suggests that AhR/ARNT pathway may be a prerequisite of chloracne pathogenesis as well (Mimura and Fujii-Kuriyama, 2003; Panteleyev and Bickers, 2006).

CYP1A1 mRNA expression is normally extremely low but is highly inducible by treatment with dioxins and related compounds. The best characterized transacting factor involved in CYP1A1 regulation is AhR. In our study, the expression level of *CYP1A1* in chloracne cases was significantly higher than that in controls, although *CYP1A1* expression was not extremely low in controls. We postulate that other factors such as smoking might contribute to this result, since most controls are smokers. Many studies revealed cigarette smoking can induce *CYP1A1* gene expression in many tissues, such as human lung and liver tissues (Chang et al., 2003; Thum et al., 2006). However, the *CYP1A1* mRNA content of the smokers was increased without significant difference with nonsmokers in the controls. The minor number of nonsmokers and differences metabolic activation of chemicals may underlie some of the expected variability.

GSTA1 is one of the glutathione-S-transferase superfamily of oxidative stress response genes. The previous studies in animal models affirmed a sustained oxidative stress response in mice following TCDD exposure. Another report on global gene expression (1152 genes) in waste incineration workers occupationally exposed to dioxin, showed upregulation of five genes related to oxidative stress (Kim et al., 2004; McHale et al., 2007).

All these results indicate that the AhR/ARNT pathway plays a critical role in the formation of chloracne. Yet, a clear proof of this assumption is still lacking. Its potential role in the pathogenesis of dioxin-induced chloracne was never assessed.

The second pathway in the action mechanism of dioxins is also the AhR-mediated but ARNT-independent activation of the Src protein kinase regulatory pathway (Enan and Matsumura, 1995; Kwon et al., 2003). Their theory is that dioxins bind to AhR, activating protein tyrosine kinase (PTK) activity, whereupon signals transmit key factor RAS protein and mitogen-activated protein kinase (MAPK) phosphorylation enzyme cascade gradually enlarge. Through MAPK phosphorylation, intranuclear activator protein-1 (AP-1) which is *c-fos* and c-jun heterodimerization, is activated. The pathway may alter the expression of a number of specific genes which are related to cell proliferation and differentiation, including plasminogen activator inhibitor-2 (PAI-2), interleukin-1 beta (IL-1 beta) (Sutter et al., 1991) and a wide range of growth factors (e.g., transforming growth factor-alpha, epidermal growth factor) (Choi et al., 1991; Davis et al., 2001). Finally TCDD results in abnormal biological effects. Thus, dioxins/AhR-induced kinase activity and protein phosphorylation pathways provide a possible molecular mechanism of chloracne.

In the present study we show that the *c-fos* transactivation level was significantly increased in epidermal tissue from chloracne lesional skin. The increase of *c-fos* expression may disturb normal proliferation and differentiation of human epidermal cells. This induction may be through AhR by increasing the Src activity pathway; the AhR/c-Src cascade may be the likely dioxin-induced signal transduction pathway leading to chloracne. However, vitro experimental results by (Hoffer et al., 1996) suggest that TCDD induces expression of the immediate-early response genes fos and jun by activation of possibly three separate signal transduction pathways, at least one of which does not require a functional Ah receptor complex.

TGF-α which is thought to act as mitogenic for keratinocytes and as an autocrine regulator of keratinocyte proliferation and differentiation (Gaido et al., 1992) is increased in TCDD-treated primary cultured human keratinocytes. This suggests that over expression of *TGF-α* is an important mechanism by which

TCDD produces its effects (Choi et al., 1991), although in our study, compared with controls, the *TGF-α* mRNA expression level of chloracne lesional skin was increased without significant difference. This may reflect the ability of TCDD to enhance *TGF-α* mRNA as transient (Davis et al., 2001) and suggests an attenuated response to long-term human exposure.

The results of this research can provide important clues with regard to dioxin toxicity. Many studies have focused on gene alterations associated with different exposures, such as Benzene and Arsensic. And most studies, including those on dioxin toxiciy, were on peripheral blood cells (Forrest et al., 2005; McHale et al., 2007; Wu et al., 2003). Our study was carried out with chloracne lesional skin, which has rarely been reported on previously. We observed that upregulation of four genes was notable in chloracne lesional skin. It is probably that constitutive activation of the AhR pathway is a prerequisite of chloracne pathogenesis. Furthermore, as in any such study, analysis of the expression of other genes in a larger population is necessary to further elucidate their roles in dioxin-induced chloracne.

4.1. Limitations

In our study, we should have obtained nonlesional skin of exposed workers at the same time for a comparison of gene expressions. However, it is very difficult to obtain consent and obtain cooperation from the workers for taking two pieces of skin from one subject, especially if one biopsy is from normal healthy skin. Moreover, a non-specific gene-expression in the lesional skin of the chloracne cases may more or less obscure our results. However, there are rarely reports about the relationship between the expression levels of the genes that we detected and other dermatologic diseases. Therefore, we did not have much theoretical evidence to refer to. This issue should be explored in further studies.

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